Antibody Reactivity to *Cryptosporidium parvum* in Saliva of Calves after Experimental Infection

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ABSTRACT. Antibodies against *Cryptosporidium parvum* in the saliva and sera of three calves experimentally infected with this parasite were examined by an indirect immunofluorescence antibody test and immunoblotting. Salivary anti-*C. parvum* IgA antibody appeared on day 12 post-challenge and had a tendency to increase transiently between days 15 and 30 post-challenge. Salivary anti-*C. parvum* IgG antibody levels showed a gradual increase along with the change in IgA antibody levels during the infection. In contrast, serum anti-*C. parvum* IgA antibody levels showed only a slight increase between days 15 and 30 post-challenge. Serum anti-*C. parvum* IgG antibody levels rose on day 12 post-challenge and one calf maintained relatively high level up to the end of the experiment. In immunoblotting, an antigen with a molecular mass of 15 kDa was found to react strongly to salivary IgA antibody and a 27 kDa antigen to react to serum IgG antibody.

KEY WORDS: *Cryptosporidium parvum*, IgA, saliva.

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Cryptosporidium parvum (*C. parvum*) causes an acute enteritis characterized by watery diarrhea in man and neonates of ruminants and has emerged as an important etiological agent of waterborne disease. The epidemiological information of *C. parvum* infection is important to prevent this disease. Immunoserological test procedures can be available to conduct immunological and seroepidemiological studies on the parasite.

Antibodies against *C. parvum* have been detected in the intestine, colostrum and blood of infected hosts [5, 7, 9]. The antibody responses in persons exposed to *C. parvum* infection was showed to differ in IgG reactivity to 15, 17 and 27 kDa *C. parvum* antigens [4]. Serum IgA antibody reactivity to the 15- and 17 kDa antigens has been recognized in several species of animals [6]. Reperant *et al.* demonstrated that the 17 kDa antigen, located on *C. parvum* sporozoites, was recognized by mucosal IgA antibodies in the early phase of infection of mice [8]. Thus, antibody responses to *C. parvum* antigens, particularly secretory IgA response to mucosal antigens, suggest that to examine the local immune response may be of interest in seroepidemiological studies.

In the present study, we investigated the IgG and IgA antibody response to *C. parvum* in the serum and saliva of calves experimentally infected with the parasites.

Oocysts of *C. parvum* used in this study were purchased from Waterborn Inc. (New Orleans, Louisiana). The oocysts were isolated from fresh feces of infected calves as described by Arrowood and Sterling [1]. Sporozoites were made

excyst from oocysts in PBS containing 0.4% sodium taurocholate and 0.2% trypsin at 37°C for 1 hr by gentle shaking and then washed three times by centrifugation at 2,000 g for 20 min in chilled PBS.

Six male Holstein-Friesian calves were obtained just after birth, without receiving colostrum, from the farm in Obihiro University of Agriculture and Veterinary Medicine. Three of them were orally inoculated with 10⁷ oocysts before 48 hr of age. Other three calves, control group were orally inoculated with 5 ml of saline. Fresh feces were collected daily and stored at 4°C. The fecal material was examined for *C. parvum* oocysts by sucrose flotation method. The serum was collected at 3-day intervals until day 33 post-inoculation and stored at -80°C until use. Saliva was absorbed in a cotton swab placed inside the calf's mouth. The swab was then put placed in a 10 ml plastic syringe, which was then placed in a 50 ml centrifuge tube, and the tube was centrifuged at 1200 × g for 15 min to recover all the liquid. The resultant supernatant was stored at -80°C until use.

The antibody reactivity to *C. parvum* in the saliva and serum of each calf was measured by an indirect immunofluorescence antibody test (IFAT). Sporozoites fixed with PBS containing 1% paraformaldehyde at 4°C for 15 min, dropped onto glass slides, dried at 4°C and stored at -80°C until use. The serum and the saliva serially diluted 4-fold and 2-fold, respectively with PBS were applied to the glass slides for 1 hr at 37°C. After washing with PBS, FITC-conjugated anti-bovine IgG (ICN Pharmaceuticals, Aurora, Ohio) or anti-bovine IgA (Bethyl Laboratories, Montgomery, Texas) diluted 200-fold with PBS, was applied to the glass slide for 1 hr at 37°C. The specific fluorescence was recognized as antibody reaction on the whole body of sporozoites under a

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Fig. 1. Mean titer fluctuation of anti-*C. parvum* antibodies in calves after experimental infection. Antibody reactivity was measured with *C. parvum* sporozoites by IFAT.

fluorescence microscope.

To determine the molecular mass of the recognized antigens, an immunoblotting assay was performed as described by Peeters *et al.* [5]. Approximately 10 mg of sporozoites suspended in 100 μ l of SDS-PAGE sample buffer (0.125 M Tris-HCl pH 6.8, 4.6% SDS, 10% 2-mercaptoethanol, 20% glycerol) was boiled for 5 min and 5 μ l of the sample was subjected to SDS-PAGE, using 12.5% acrylamide gel, and transblotted onto a polyvinylidene difluoride microporous membrane (Millipore Inc., Tokyo, Japan). After blocking in PBS containing 3% bovine serum albumin (PBS-BSA) at 4 °C for 18 hr, the membrane was immersed in either 100-fold diluted test sera or 20 fold diluted test saliva at 4°C for 18 hr. The membrane was allowed to react with the secondary antibody that is either horseradish peroxidase-conjugated anti-bovine IgG or horseradish peroxidase conjugated anti-bovine IgA (both obtained from Bethyl Laboratories, Montgomery, Texas) 1,000-fold diluted in PBS-BSA, at 4°C for 18 hr. The antibody was detected by means of the peroxidase reaction

using diaminobenzidine-4HCl in 0.1 M Tris-HCl at pH 7.4 containing 0.03% H₂O₂. Molecular masses of electrophoresed proteins were estimated from their running distance compared with that of molecular mass markers (broad range molecular weight markers; Bio-Rad. Ltd, Richmond, California).

All calves inoculated orally with *C. parvum* oocysts developed watery diarrhea between days 3 and 10 post-challenge. Oocyst shedding started from day 4 post-challenge and the fecundity of shedding was more than 10⁸ oocysts per 1 ml of feces between days 5 and 7. Then, the oocyst shed gradually decreased in number to below 10⁵/ml but continuously passed for 4 weeks post-challenge.

Figure 1 shows the mean anti-*C. parvum* IgA and IgG titers in the saliva of the calves tested. IgA antibodies appeared in the saliva of all the inoculated calves on day 12, and had a tendency to increase between days 15 and 30 post-challenge. Thereafter the titers decreased to the control level (1: 2). IgG antibodies also appeared in the saliva on day 12

Fig. 2a. Immunoblotting of *C. parvum* antigens reacting to IgA antibodies in either saliva and serum from calves after experimental infection. Numbers in the left margin indicate molecular mass. Lane numbers indicate the day post-challenge when sampling was performed in infected calves, or the day after birth in controls. Arrows indicate 15 and 27 kDa antigens.

Fig. 2b. Immunoblotting of *C. parvum* antigens reacting to IgG antibodies in either saliva and serum from calves after experimental infection. Numbers in the left margin indicate molecular mass. Lane numbers indicate the day post-challenge when sampling was performed in infected calves, or the day after birth in controls. Arrows indicate 15 and 27 kDa antigens.

and the titers increased gradually during the experimental period up to day 33 post-challenge (1: 64) in one of the 3 calves. IgG antibodies against *C. parvum* also appeared in the serum of 2 of the 3 inoculated calves on day 12 post-challenge (1: 16) and the titers remained relatively high up to the end of the experiment (Fig. 1). Anti-*C. parvum* IgA antibody was detected in serum (calf 1; 1:16, calf 2 and 3; 1:8) between days 12 and 24 post-challenge.

In immunoblotting with IgA antibody, more than 10 bands

were recognized as *C. parvum* antigens in the saliva and serum of infected calves between days 12 and 30 post-challenge (Fig. 2a). Particularly, IgA antibody in the saliva showed higher reactivity to an antigen with a molecular mass of approximately 15 kDa than to the others. IgG antibody in the saliva showed relatively weak reactivity to the parasite antigens, even though the IFAT titer was similar in both antibodies. In the serum of one infected calf, IgG antibody showed high reactivity to 27 kDa antigen (Fig. 2b). No spe-

cific bands were detected in the assays of the antibody reactivity in saliva and serum samples from non-infected calves.

Cozon *et al.* demonstrated that AIDS patients with chronic cryptosporidiosis had an increased level of anti-*C. parvum* secretory IgA in their saliva, compared to control subjects [2]. In this study, all the three calves experimentally infected with *C. parvum* showed a transient increase in the level of anti-*C. parvum* IgA antibody in saliva in the convalescent phase of the infection, although the anti-*C. parvum* IgA antibody level in the serum was not higher than that in the saliva. A similar observation regarding the specific IgA response in intestinal secretions of lambs has been reported [3]. The wet weight of intestinal secretion and fecal materials varies, however, dependent on the water content of the samples which obtained may contain IgA secreted through hepatobiliary ducts and/or serum antibodies derived from the blood leaked into the intestine by breakage of the intestinal mucosa. Our observations suggest that the level of anti-*C. parvum* IgA antibody in saliva may reflect the immune response after the primary infection and that saliva samples may be available for analysis of antibody production responding to *C. parvum* antigens. Interestingly, IgA antibody present in the saliva in the convalescent phase of infection recognized a protein with a molecular mass of approximately 15 kDa, as the predominant antigen. Tilley *et al.* [8] also found that a 15 kDa glycoprotein was the most prominent, and monoclonal antibodies against this antigen recognized epitopes on sporozoites and

merozoites but not on the sexual-stages of the parasite. It is likely that the 15 kDa antigen may be a useful marker for discriminating between *C. parvum* and other pathogens.

Further studies should be carried out to determine the functional role of the 15 kDa antigenic component.

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